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LincRNA1230 inhibits the differentiation of mouse ES cells towards neural progenitors

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In vitro, mouse embryonic stem (ES) cells can differentiate into many somatic cell types, including neurons and glial cells. When cultured in serum-free medium, ES cells convert spontaneously and efficiently to a neural fate. Previous studies have shown that the neural conversion of mouse ES cells includes both the participation of neural-specific transcription factors and the regulation of epigenetic modifications. However, the intracellular mechanism underlying this intrinsic transition still remains to be further elucidated. Herein, we describe a long intergenic non-coding RNA, *LincRNA1230*, which participates in the regulation of the neural lineage specification of mouse ES cells. The ectopic forced expression of *LincRNA1230* dramatically inhibited mouse ES cells from adopting a neural cell fate, while *LincRNA1230* knockdown promoted the conversion of mouse ES cells towards neural progenitors. Mechanistic studies have shown that *LincRNA1230* inhibits the activation of early neural genes, such as *Pax6* and *Sox1*, through the modulation of bivalent modifications (tri-methylation of histone3 lysine4 and histone3 lysine27) at the promoters of these genes. The interaction of *LincRNA1230* with *Wdr5* blocked the localization of *Wdr5* at the promoters of early neural genes, thereby inhibiting the enrichment of H3K4me3 modifications at these loci. Collectively, these findings revealed a crucial role for *LincRNA1230* in the regulation of the neural differentiation of mouse ES cells.

mouse ES cells, neural differentiation, long non-coding RNA (lncRNA), bivalent modification, *Wdr5*

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INTRODUCTION

Mouse ES cells are initially derived from pre-implanted blastocysts (Evans and Kaufman, 1981) and have the ability to develop into all three germ lines (Ying et al., 2003). *In vitro*, ES cells can differentiate into various somatic cell types with specific biological functions, such as myocardial cells (Siu et al., 2007), pancreatic β cells (Pagliuca et al., 2014), neurons (Kamiya et al., 2011; Selvaraj et al., 2012; Watanabe et al., 2005) and glial cells (Selvaraj et al., 2012). When cultured in a serum-free medium without inhibitory environmental signals, ES cells form floating aggregates (serum-free culture of embryoid-body like aggregates, known as SFEB) and efficiently commit into a neural fate in a so-called "default model" (Kamiya et al., 2011; Watanabe et al., 2005). The establishment of the *in vitro* neural differentiation system has provided insight into the mechanisms underlying the nervous system development of vertebrates.

Using this serum-free neural differentiation model, scientists have revealed the regulatory function of many transcription factors (TFs), such as *Zfp521* and *Oct6*, which play important roles in the conversion of mouse ES cells into neural progenitors. *Zfp521* interacts with the co-activator *P300* to initiate the expression of several early neural genes, such as *Sox1*, *Sox3*, and *Pax6* (Kamiya et al.,

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2011). *Oct6* activates neural differentiation-associated genes and represses the downstream targets of BMP and WNT signaling pathways to facilitate neural lineage commitment (Zhu et al., 2014). These studies have provided new insights into the mechanism of neural fate specification, however, it remains unknown how the expression of these TFs is precisely activated along with the initiation of neural conversion.

In addition to TFs, epigenetic regulations, particularly histone modifications, have also been implicated in the neural differentiation of ES cells (Ziller et al., 2015). In undifferentiated ES cells, many developmental genes are in a "poised" state, with the enrichment of both the repressive epigenetic marker tri-methylation of histone3 lysine27 (H3K27me3) and the active epigenetic marker tri-methylation of histone3 lysine4 (H3K4me3) at the promoters of these developmental genes. The co-localization of H3K27me3 and H3K4me3 is called "bivalent modification" (Bernstein et al., 2006a; Vastenhouw and Schier, 2012b). Genes with bivalent modifications are silenced or in a low expression state until differentiation is initiated (Bernstein et al., 2006b). Many epigenetic modifiers have been implicated in the regulation of the bivalent modifications of developmental genes. For example, the PRC2 complex catalyzes the tri-methylation of H3K27 (Cooper et al., 2014), and the Trithorax complex catalyzes the tri-methylation of H3K4 (Denissov et al., 2014; Schuettengruber et al., 2011). Research has shown that many neural lineage-associated genes, such as *Pax6, Sox1, Sox3* and *Nestin*, are under bivalent modifications (Hirabayashi and Gotoh, 2010). During neural differentiation, the transcriptional activation of these poised genes requires both reduced H3K27me3 and increased H3K4me3 histone modifications (Jiang et al., 2011). Studies have shown that the attenuation of H3K4me3 modification through the interference of core Trithorax complex components, such as *Dpy-30* and *RbBP5*, inhibits RA-induced neural differentiation (Jiang et al., 2011).

Defined as genomic transcripts longer than 200nt with no protein coding potential (Ma et al., 2013), long non-coding RNAs (lncRNAs) participate in many biological processes, including embryonic development, cell differentiation (Rinn et al., 2007a) and proliferation (Sun et al., 2015) and have even been implicated in some pathological processes, such as tumorigenesis (Park et al., 2014; Shore et al., 2012; Wu et al., 2015). Studies have shown that the perturbation of certain lncRNAs in ES cells results in repression of pluripotency-associated genes or elevated expression of development-associated genes, indicating that lncRNAs are highly associated with the maintenance of ES cell pluripotency and the conversion of its differentiation potential tendencies (Guttman et al., 2011; Wang et al., 2013). LncRNAs involved in neural lineage commitment have also been reported (Sauvageau et al., 2013). LncRNA *TUNA* recruits *Sox2* to downstream neural genes to regulate the differentiation of mouse ES cells toward neural progenitors (Lin et al., 2014). In addition, *RMST* physically interacts with *Sox2* and is required for the binding of *Sox2* to promoters of neurogenic TFs (Ng et al., 2013).

The expression of *LincRNA1230*, a long intergenic non-coding RNA (lincRNA), was affected during the neural differentiation of mouse ES cells according to large-scale microarray data (Guttman et al., 2011), but the function of this molecule has not been investigated. The results of the present study showed that the forced expression of *LincRNA1230* strongly inhibited the transition of mouse ES cells into neural progenitors in SFEB culture, while the knockdown of *LincRNA1230* promoted this process. Mechanistically, *LincRNA1230* inhibited the enrichment of the H3K4me3 modification through interaction with *Wdr5*, a core component of the Trithorax complex, thereby blocking the binding of *Wdr5* to the promoter regions of neural lineage-associated genes.

RESULTS

Differential expression patterns of *LincRNA1230* during *in vitro* mouse ES cell differentiation

We used an ES cell line with GFP knocked in at the *Sox1* locus (Aubert et al., 2003) to perform SFEB neural differentiation. The results of the Q-PCR analysis showed decreased expression of *Rex1* and gradually elevated expression of neural marker genes, such as *Zfp521*, *Nestin* and *Sox1* during seven days of SFEB culture (Figure 1A). The results of the FACS assay showed a high *Sox1*-positive cell ratio (65.4%±0.7%) after seven days of differentiation (Figure 1B). In addition, immunostaining also demonstrated the presence of *Sox1*- and *N-cad*-positive neural progenitors (Figure 1C). These results suggested that the neural differentiation system used in the present study was valid and efficient. We subsequently examined the expression of several lincRNAs that are predicted to be associated with neural ectoderm differentiation in previous studies (Guttman et al., 2011) during neural differentiation. The Q-PCR analysis showed that the expression of *LincRNA1230* (Figure S1 in Supporting Information) is gradually decreased during neural differentiation. After seven days of differentiation, the expression level of *LincRNA1230* decreased by approximately 80% (Figure 1D). To determine whether *LincRNA1230* exhibited similar expression pattern during mesendodermal differentiation, we performed embryoid body (EB) formation using 15% fetal bovine serum (FBS), which differentiated mouse ES cells into a mesendodermal cell fate. Q-PCR showed decreased expression of *Rex1* and elevated expression of mesendodermal marker genes, such as *T*, *Gata4*, and *Gata6*, during seven days of EB formation culture (Figure 1E). Immunostaining showed large percent of *T*- and *Gata4*-positive cells after five days of differentiation (Figure 1F), while FACS analysis detected few *Sox1*-positive cells (3.8%±0.3%, Figure 1G). These results validated the feasibility of the mesendodermal differentia-

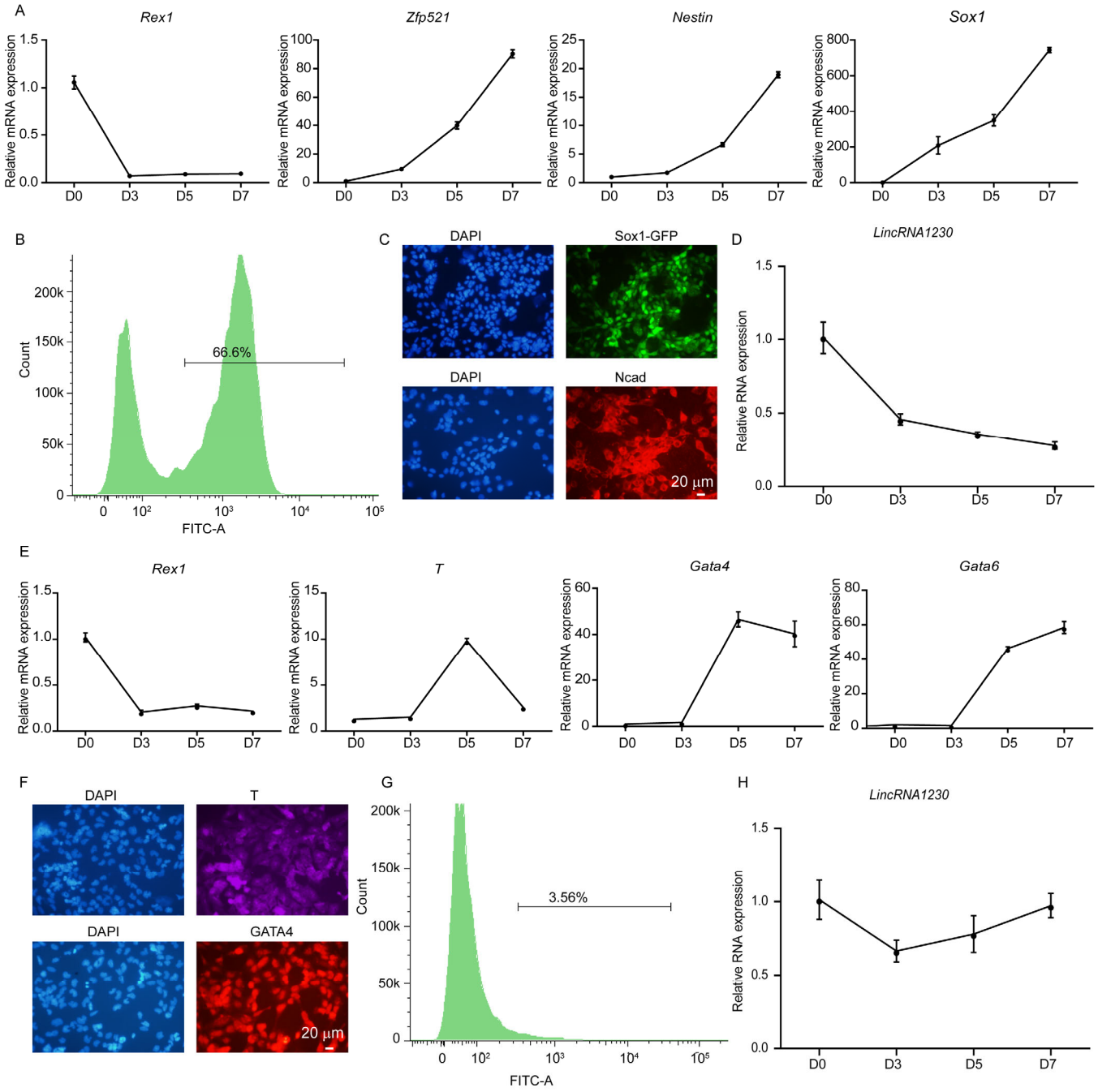

Figure 1 *LincRNA1230* is specifically down regulated during neural differentiation. A, Q-PCR verification of mRNA levels of pluripotent (*Rex1*) and neural lineage-associated (*Zfp521*, *Nestin*, and *Sox1*) genes during SFEB neural differentiation culture. The data are shown as the means±SEM (n=3). B, FACS assay showing the positive ratio of *Sox1*-GFP cells after seven days of SFEB culture. C, Immunofluorescence analysis of *Sox1*-GFP (green) and *Tuj1* (red) indicate the generation of neural progenitors after seven days of SFEB differentiation. 4′,6-diamidino-2-phenylindole (DAPI) represents nuclear staining (blue). D, Q-PCR assay to investigate the expression of *LincRNA1230* during neural differentiation. The data are shown as the means±SEM (n=3). E, Q-PCR verification of the mRNA levels of pluripotent (*Rex1*) and mesendodermal lineage-associated (*T*, *Gata4*, and *Gata6*) genes during EB formation differentiation culture. The data are shown as the means±SEM (n=3). F, Immunofluorescence analysis of *T* (purple) and *Gata4* (red) to indicate the generation of mesendodermal lineage cells after five days of differentiation. DAPI represents nuclear staining (blue). G, FACS assay showing the positive ratio of *Sox1*-GFP cells after seven days of EB formation culture. H, Q-PCR assay to investigate the expression pattern of *LincRNA1230* during mesendodermal differentiation. The data are shown as the means±SEM (n=3).

tion system. Even though a slight reduction of *LincRNA1230* expression was detected in differentiation day three, no substantial change in the expression of *LincRNA1230* was detected after seven days of mesendodermal differentiation compared to undifferentiated ES cells (Figure 1H). These results demonstrated that *LincRNA1230* is

specifically down regulated during neural differentiation, indicating that the suppressed expression of *LincRNA1230* might be required for the initiation of neural lineage commitment.

Overexpression of *LincRNA1230* inhibits the neural differentiation of mouse ES cells

As *LincRNA1230* is down regulated during SFEB culture, we examined whether the decreased expression of *LincRNA1230* is required for neural lineage commitment. We established a *LincRNA1230* overexpression cell line and validated the efficiency of overexpression using Q-PCR analysis. The results showed that the expression of *LincRNA1230* was elevated more than 15-fold in both ES cells and during neural differentiation (Figure 2A). The ectopic forced expression of *LincRNA1230* showed no substantial influence on the pluripotency maintenance of ES cells, as the *LincRNA1230*-overexpressing ES cell line manifested normal ES cell clone morphology and exhibited positive alkaline phosphatase (AP) staining (Figure S2A, Line 1 in Supporting Information). Western blot analysis also detected comparable expression levels of pluripotent marker genes, such as *Oct4*, *Nanog*, and *Sox2* (Figure S2B, Lines 1 and 2 in Supporting Information). Fluorescence microscopy observation showed strongly suppressed *Sox1-GFP* expression with overexpression of *LincRNA1230* after seven days of neural induction (Figure 2B). FACS analysis demonstrated that the overexpression of *LincRNA1230* dramatically decreased the *Sox1*-positive cell ratio (*LincRNA1230* overexpression vs. control: 66.5%±1.0% vs. 29.7%±1.3%) after seven days of SFEB culture (Figure 2C) and also suppressed the expression of other neural progenitor makers, such as *Pax6*, *Zfp521*, *Sox2*, *Sox1*, *Nestin* and *Tuj1* (Figure 2D). In addition, immunostaining showed the reduced generation of *Sox1*- and *Nestin*-positive neural progenitors (Figure 2E). To determine whether *LincRNA1230* is involved in mesendodermal differentiation, we subjected the *LincRNA1230*-overexpressing cell line to EB formation and examined the expression of mesendodermal marker genes, such as *Gata4*, *Gata6*, T, *Eomes*, *GSC*, *AFP*, *Mixl1*, and *SMA* using Q-PCR. The forced expression of *LincRNA1230* showed no substantial effect on the expression of these genes (Figure 2F), indicating that the inhibitory effect of *LincRNA1230* on differentiation is neural lineage-specific. Thus, these results demonstrated that *LincRNA1230* overexpression strongly and specifically inhibited the transition of mouse ES cells to neural progenitors.

Knockdown of *LincRNA1230* promotes the neural differentiation of mouse ES cells

We subsequently investigated whether the interference of *LincRNA1230* facilitates neural differentiation. We established a doxycycline-inducible *LincRNA1230* knockdown ES cell line and validated the knockdown efficiency through Q-PCR. The results showed that the addition of doxycycline decreased the expression of *LincRNA1230* by 80% in ES cells, and the perturbation effect persisted during the seven days of SFEB culture (Figure 3A). Consistently, the knockdown of *LincRNA1230* also showed no substantial influence on the pluripotency maintenance of ES cells (Figure S2A, Line 2; S2B, Lines 3 and 4 in Supporting Information). Fluorescent microscopy showed the enhanced expression of *Sox1-GFP* with the addition of doxycycline after seven days of neural differentiation (Figure 3B). FACS analysis also showed that the knockdown of *LincRNA1230* increased the ratio of Sox1-positive cells (*LincRNA1230* knockdown vs. control: 64.6%±2.1% vs. 82.3%±1.4%, Figure 3C). We subsequently examined the expression of other neural progenitor markers using Q-PCR, and the results showed enhanced expression of *Pax6*, *Zfp521*, *Sox2*, *Nestin* and *Tuj1* (Figure 3D). Immunostaining also showed increased *Sox1*- and *Nestin*-positive cells with the addition of doxycycline (Figure 3E). Indeed, the addition of doxycycline during the first three days of neural differentiation could efficiently promote the generation of *Sox1*-positive neural progenitors (from 63.7%±1.1% to 75.3%±1.5%, Figure 3F). In the EB formation system, the knockdown of *LincRNA1230* showed no obvious effect on the expression of mesendodermal genes (Figure 3G), consistent with the results observed using the *LincRNA1230*-overexpressing cell line. These results demonstrated that the knockdown of *LincRNA1230* could specifically facilitate the neural lineage commitment of mouse ES cells. Taken together, these results suggested that *LincRNA1230* acts as a barrier that specifically attenuates the neural differentiation of mouse ES cells in SFEB culture. The expression of *LincRNA1230* in ES cells prevents them from undergoing spontaneous neural fate specification, thereby maintaining the pluripotency of ES cells.

LincRNA1230 regulates the bivalent modification of neural genes

We next investigated the mechanism underlying the regulatory function of *LincRNA1230* on neural differentiation. To this end, we examined whether *LincRNA1230* could affect the activity of developmental genes in mouse ES cells. Q-PCR analysis demonstrated that the forced expression of *LincRNA1230* attenuated the expression of neural lineage-associated developmental genes, such as *Sox1*, *Pax6* and *Zic2* (Figure 4A), while the knockdown of *LincRNA1230* enhanced the expression of *Sox1* and *Pax6* (Figure 4B). Collectively, these results demonstrated that *LincRNA1230* was involved in the regulation of the activation of early neural genes in mouse ES cells. As many developmental genes in ES cells are under bivalent modification, we next examined whether *LincRNA1230* could influence H3K4me3/H3K27me3 modifications at the promoters of these genes. A ChIP assay showed that the forced expression of *LincRNA1230* suppressed the enrichment of H3K4me3 modification at the promoters of early neural

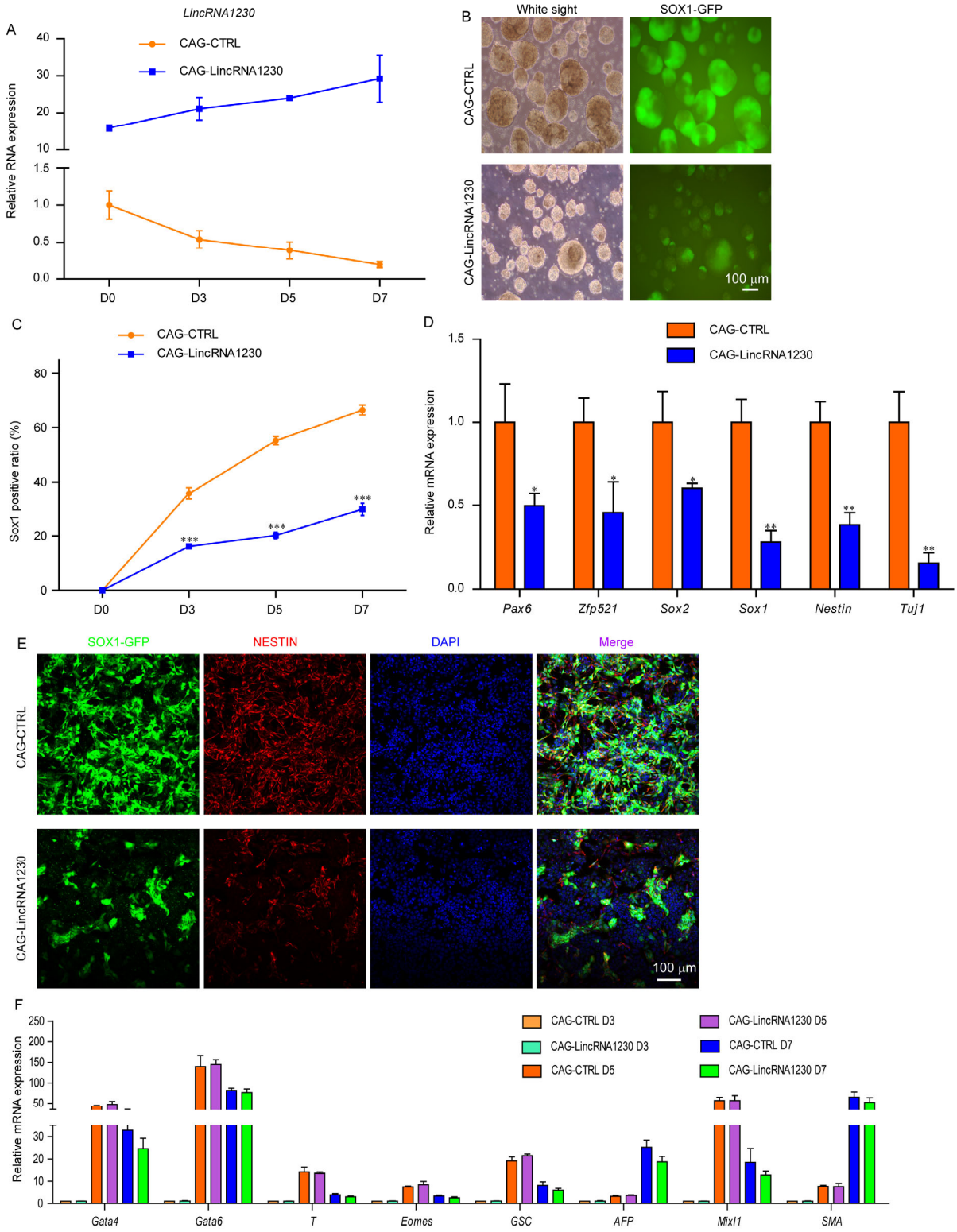

Figure 2 Overexpression of *LincRNA1230* inhibits the differentiation of mouse ES cells into neural progenitors. A, Q-PCR verification of the overexpression of *LincRNA1230* during the neural transition of mouse ES cells. The data are shown as the means±SEM (n=3). B, Fluorescence microscopy of the *Sox1*-GFP-positive SFEB aggregates with *LincRNA1230* overexpression. C, FACS assay showing the positive ratio of *Sox1*-GFP cells during seven days of SFEB culture with *LincRNA1230* overexpression. The data are shown as the means±SEM (n=3). ***, P<0.001. D, Q-PCR assay showing the expression of neural progenitor marker genes after seven days of neural differentiation with *LincRNA1230* overexpression. The data are shown as the means±SEM (n=3). *, P<0.05. **, P<0.01. E, Immunofluorescence analysis of *Sox1*-GFP (green) and *Nestin* (red) to indicate the generation of neural progenitors after seven days of differentiation with *LincRNA1230* overexpression. DAPI represents cell nuclear staining (blue). F, Q-PCR assay showing the expression of mesendodermal marker genes during seven days of EB formation culture with *LincRNA1230*overexpression. The data are shown as the means±SEM (n=3).

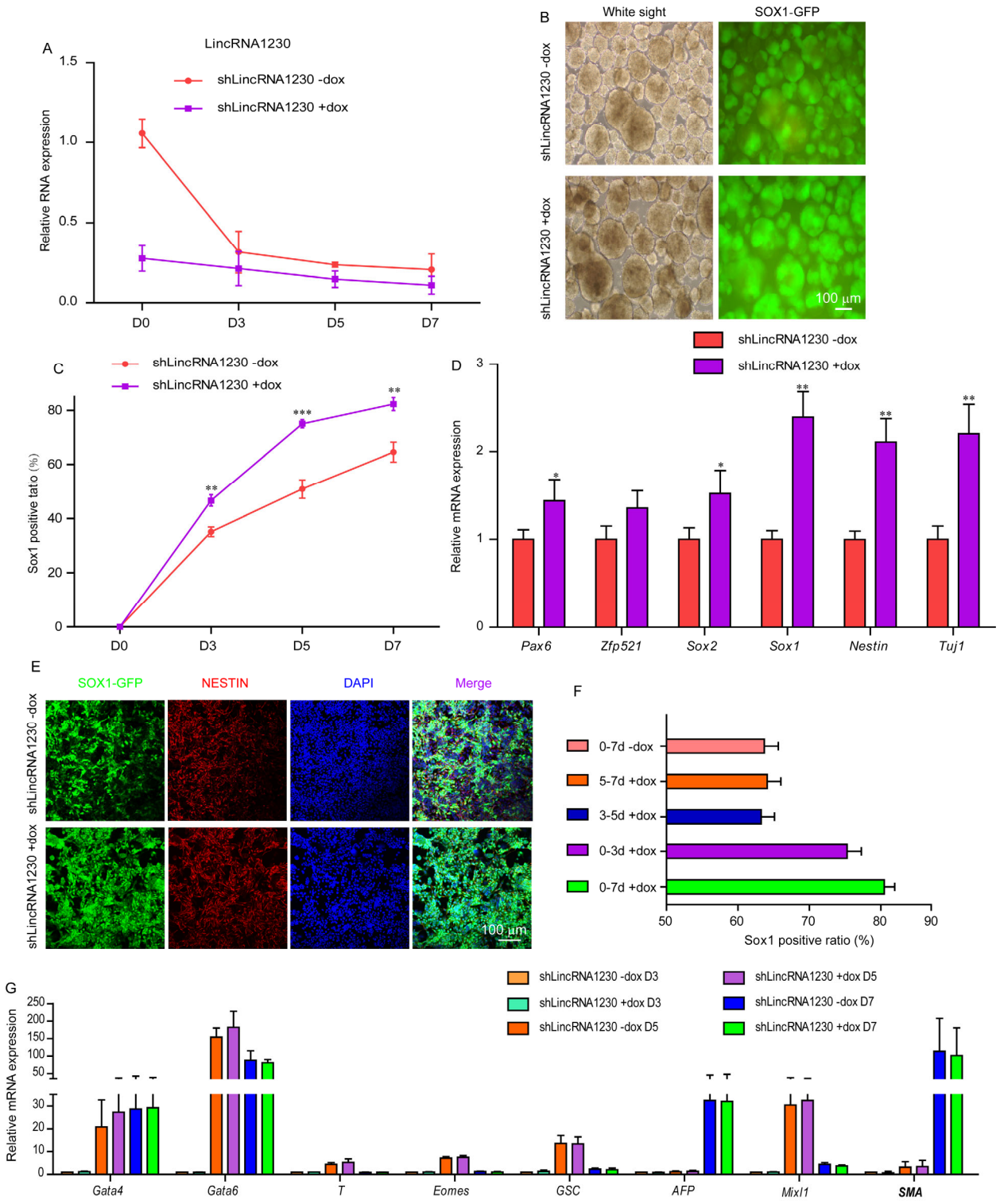

Figure 3 Knockdown of *LincRNA1230* promotes the differentiation of mouse ES cells into neural progenitors. A, Q-PCR verification of the knockdown of *LincRNA1230* during the neural transition of mouse ES cells. The data are shown as the means±SEM (n=3). B, Fluorescence microscopy of the *Sox1*-GFP-positive SFEB aggregates with *LincRNA1230* knockdown. C, FACS assay showing the positive ratio of *Sox1*-GFP cells during seven days of SFEB culture with *LincRNA1230* knockdown. The data are shown as the means±SEM (n=3). **, P<0.01, ***, P<0.001. D, Q-PCR assay showing the expression of neural progenitor marker genes after seven days of neural differentiation with *LincRNA1230* knockdown. The data are shown as the means±SEM (n=3). *, P<0.05. **, P<0.01. E, Immunofluorescence analysis of *Sox1*-GFP (green) and *Nestin* (red) to indicate the generation of neural progenitors after seven days of differentiation with *LincRNA1230* knockdown. DAPI represents nuclear staining (blue). F, FACS assay showing the positive ratio of *Sox1*-GFP cells after seven days of SFEB culture with the addition of doxycycline at various time points. The data are shown as the means±SEM (n=3). G, Q-PCR assay showing the expression of mesendodermal marker genes during seven days of EB formation culture with *LincRNA1230* knockdown. The data are shown as the means±SEM (n=3).

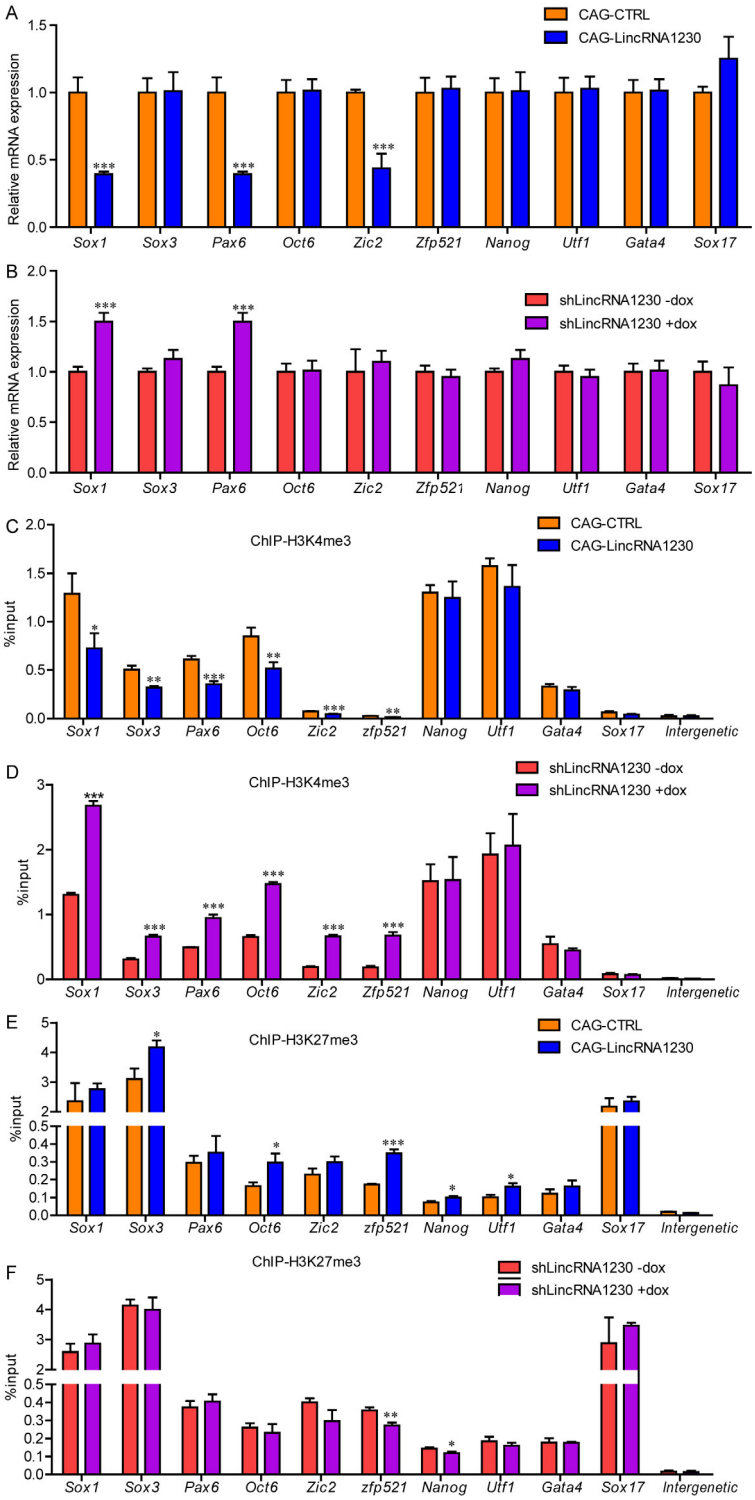

Figure 4 *LincRNA1230* inhibits the enrichment of H3K4me3 at the promoters of neural differentiation-associated genes. A, Q-PCR assay showing the expression of developmental and pluripotent genes in mouse ES cells after *LincRNA1230*overexpression. Data shown are means±SEM (n=3).***, P<0.001. B, Q-PCR assay shows the expression of developmental genes and pluripotent genes in mouse ES cells after the knockdown of *LincRNA1230*. Data shown are means±SEM (n=3).***, P<0.001. C, ChIP assay showing the enrichment of H3K4me3 at promoters of developmental and pluripotent genes in mouse ES cells after the overexpression of *LincRNA1230*. Data shown are means±SEM (n=3). *, P<0.05. **, P<0.01. ***, P<0.001. D, ChIP assay showing the enrichment of H3K4me3 at the promoters of developmental and pluripotent genes in mouse ES cells after *LincRNA1230* knockdown. The data are shown as the means±SEM (n=3). ***, P<0.001. E, ChIP assay showing the enrichment of H3K27me3 at the promoters of developmental and pluripotent genes in mouse ES cells after *LincRNA1230* overexpression. The data are shown as the means±SEM (n=3). *, P<0.05. ***, P<0.001. F, ChIP assay showing the enrichment of H3K27me3 at the promoters of developmental and pluripotent genes in mouse ES cells after *LincRNA1230* knockdown. The data are shown as the means±SEM (n=3). *, P<0.05. **, P<0.01.

genes, including *Sox1*, *Sox3*, *Pax6*, *Oct6*, *Zic2* and *Zfp521*, but had minor effects on pluripotency genes, such as *Nanog* and *Utf1,* or mesendodermal genes, such as *Gata4* and *Sox17* (Figure 4C). ChIP assay also detected that the knockdown of *LincRNA1230* enhanced the enrichment of H3K4me3 at the promoters of these early neural genes (Figure 4D). In addition, we also detected increased H3K27me3 modification at the promoters of *Sox3*, *Pax6* and *Zfp521* with forced expression of *LincRNA1230* (Figure 4E) and attenuated H3K27me3 at the promoter of *Zfp521* in *LincRNA1230* knockdown ES cells (Figure 4F). These results demonstrated that *LincRNA1230* influences the poised status of early neural genes through the inhibition of H3K4me3 enrichment at the promoters of early neural genes. The decreased H3K4me3 modification maintains neural genes at a more "blunt" status so that they cannot be appropriately activated in response to neural differentiation cues.

LincRNA1230 blocks the enrichment of *Wdr5* at the promoters of neural genes

Studies have shown that the bivalent modifications of developmental genes are regulated through both the PRC2 and Trithorax complexes. To determine whether these two histone modification complexes participate in *LincRNA1230*-mediated neural differentiation, we performed an RNA immunoprecipitation assay to detect the interaction of *LincRNA1230* with components of these two complexes, such as *Ezh2*, *Suz12* (members of the PRC2 complex) and *Wdr5* (a member of the Trithorax complex). The results demonstrated that *LincRNA1230* physically interacts with the Trithorax complex component *Wdr5* (>10-fold enrichment compared with IgG), while no strong interaction with *Suz12* or *Ezh2* was observed (Figure 5A), indicating that *LincRNA1230* might participate in the regulation of H3K4me3 through interactions with *Wdr5*. *Wdr5* was reported to interact with thousands of RNA transcripts through its RNA-binding domain (Yang et al., 2014), and the interaction of *Wdr5* with RNA transcripts might influence the stability of this protein. A western blot analysis showed that neither the overexpression nor the knockdown of *LincRNA1230* had a substantial effect on the protein level of *Wdr5* (Figure 5B). Moreover, we examined the relationship between the *Wdr5-LincRNA1230* interaction and the regulation of H3K4me3 modification through *LincRNA1230*. The results of the ChIP assay demonstrated that the overexpression of *LincRNA1230* strongly inhibited the enrichment of *Wdr5* at neural gene promoters (Figure 5C), while the knockdown of *LincRNA1230* facilitated the enrichment of *Wdr5* at these regions (Figure 5D). These results suggested that the interaction of *LincRNA1230* with *Wdr5* prevents the binding of *Wdr5* to promoters of key neural developmental genes, thus attenuating the enrichment of H3K4me3 modification at these promoters. *LincRNA1230* regulates the neural differentiation potential of mouse ES cells by modulating the H3K4me3 modification at the promoters of key neural developmental genes through interactions with *Wdr5* (Figure 5E).

DISCUSSION

Neural induction is initiated at the early stages of embryogenesis. Developmental disorders that occur during neural induction might result in various types of congenital diseases, including aepnenchaly (Gole et al., 2014) and cerebral palsy (Panteliadis et al., 2015). Thus, the regulation of neural lineage determination must be under precise control to guarantee the normal development of the nervous system. During embryonic development and ES cell differentiation, precise neural fate specification depends on the coordination of allowable extracellular signals and proper intracellular environments. Previous studies have revealed various inhibitory or active cues that participate in the regulation of early neural differentiation (Kamiya et al., 2011; Koshida et al., 2002; Louvi and Artavanis-Tsakonas, 2006; Patapoutian and Reichardt, 2000; Zhu et al., 2014). However, the involvement of lncRNAs in neural induction has rarely been reported. In the present study, we revealed the important regulatory function of the lincRNA, *LincRNA1230*, in regulating the neural conversion of mouse ES cells. *LincRNA1230* dramatically inhibits the conversion of mouse ES cells into neural progenitors, demonstrating that *LincRNA1230* most likely acts as a switch in the neural fate determination of mouse ES cells.

In the serum-free differentiation system used in the present study, extracellular cues were minimized, thus for further investigation, we focused on the influence of *lincRNA1230* on genetic transcription activities and epigenetic modifications. The Q-PCR data showed that *lincRNA1230* inhibited the expression of some early neural developmental genes, and many of which are under bivalent modification (Hirabayashi and Gotoh, 2010). Bivalent histone modification occurs at the promoters of many developmental genes in ES cells and plays an important role in cell fate determination (Vastenhouw and Schier, 2012a). Disordered bivalent histone modification in mouse ES cells might lead to the failure of neural conversion. Studies have reported that theattenuation of H3K4me3 modification by interference of core Trithorax complex components such as *Dpy-30* and *RbBP5* caused inhibition of RA induced neural differentiation (Jiang et al., 2011). However, it is still unclear how this important histone modification pattern is regulated in response to various developmental cues. In the present study, we identified *LincRNA1230* as a modifier of bivalent modification, which inhibits the enrichment of H3K4me3 at the promoters of neural developmental genes. The expression of *LincRNA1230* in mouse ES cells maintained the balance of H3K4me3/H3K27me3 modifications at the promoters of neural lineage-associated genes to ensure that these genes are maintained in a poised status. Once neural conversion is

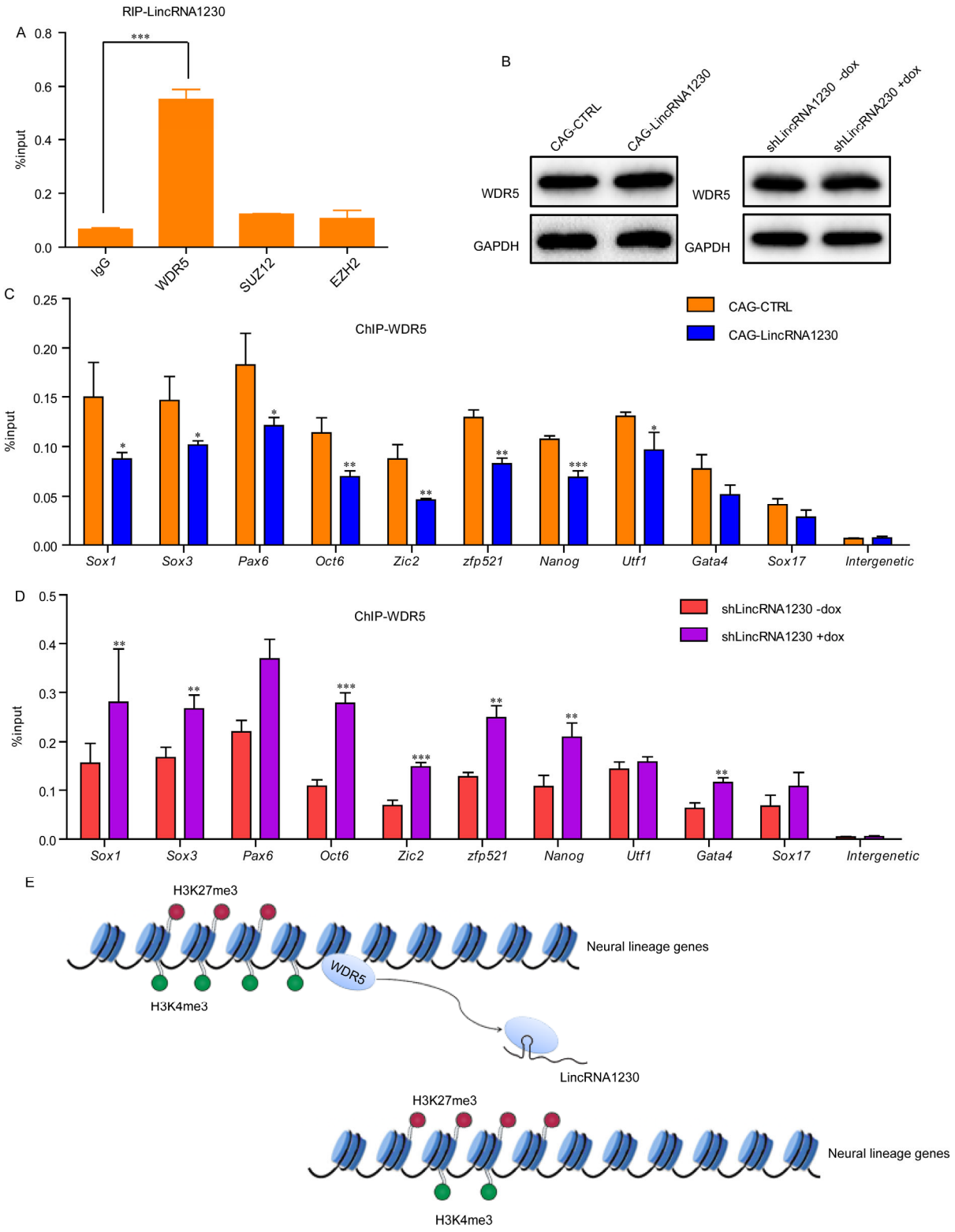

Figure 5 *LincRNA1230* blocks the enrichment of *Wdr5* at the promoters of neural differentiation-associated genes. A, RIP assay showing the interaction of *LincRNA1230* with the Trithorax complex component *Wdr5*. The data are shown as the means±SEM (n=3). ***, P<0.001. B, Westernblot showing the expression of *Wdr5* with the overexpression or knockdown of *LincRNA1230* in mouse ES cells. C, ChIP assay showing the enrichment of *Wdr5* at the promoters of developmental and pluripotent genes in mouse ES cells after *LincRNA1230* overexpression. The data are shown as the means±SEM (n=3). *, P<0.05. **, P<0.01. ***, P<0.001. D, ChIP assay showing the enrichment of *Wdr5* at the promoters of developmental and pluripotent genes in mouse ES cells after *LincRNA1230* knockdown. The data are shown as the means±SEM (n=3). **, P<0.01. ***, P<0.001. E, Schematic diagram shows that *LincRNA1230* participates in the regulation of mouse ES cell differentiation tendency towards neural lineage.

initiated, the expression of *LincRNA1230* is attenuated so that the H3K4me3 modification is enriched at the promoters of early neural genes to convert these genes from a poised state to an active status.

Many lncRNAs have been implicated in epigenetic modification. Previous studies have indicated that, in most cases, lncRNAs function through guiding specific epigenetic modifiers to targeted genomic loci (Batista and Chang, 2013; Guttman and Rinn, 2012). *HOTTIP* regulates the formation of the active H3K4me3 mark by recruiting Trithorax (Wang et al., 2011), and *HOTAIR* functions as a scaffold for the recruitment of two distinct repressive complexes, PRC2 and Rest, on *HoxD* genes (Rinn et al., 2007b). In the present study, we proposed that *LincRNA1230* participates in the regulation of H3K4me3 at bivalent genes in a different manner: the physical interaction of *LincRNA1230* with the Trithorax complex component *Wdr5* blocked the localization of *Wdr5* at the promoters of neural lineage-associated genes, thus inhibiting the enrichment of H3K4me3 at these loci. ChIP-qPCR data indeed demonstrated the attenuated enrichment of the H3K4me3 modification and inhibited the localization of *Wdr5* specifically on certain neural lineage-associated genes with the overexpression of *LincRNA1230*. These findings revealed a distinct mechanism of lncRNAs in modulating epigenetic modifications. Nonetheless, it remains unknown how the specificity of the regulatory function of *LincRNA1230* for targeting neural lineage-associated genes is achieved and whether there are other factors that are involved in the *LincRNA1230-Wdr5* interaction and confer this complex targeting specificity.

In summary, the findings of the present study established *LincRNA1230* as a critical regulator of bivalent histone modifications for the poised transition of mouse ES cells towards neural progenitors. Thus, these results further revealed the interaction of TFs, histone modification complexes and non-coding RNAs in cell fate determinations and complemented the regulatory circuitry of neural lineage commitment.

MATERIALS AND METHODS

Cell culture and differentiation

ES cells were cultured on a feeder layer produced using irradiated mouse embryonic fibroblasts (MEFs) in Dulbecco's modified eagle medium (DMEM) medium (Gibco, USA) containing 15% fetal bovine serum (FBS; Gibco), 0.1 mmol L^{-1} β-mercaptoethanol (Sigma, USA), 2 mmol L^{-1} glutamine (Invitrogen, USA), 2 mmol L^{-1} nonessential amino acids (Invitrogen), 1 mmol L^{-1} sodium pyruvate (Invitrogen), and 20 ng mL^{-1} homemade LIF. For cell passage, the ES cells were digested with 0.05% trypsin-edetic acid (EDTA) (Gibco) at 37°C for 2 min and subsequently seeded onto 6-well plates with 1×10^5 cells per well. For neural differentiation, ES cells were suspended to form aggregates in Glasgow's minimum essential medium (GMEM) (Gibco) supplemented with 8% knockout serum replacement (KOSR; Gibco), 0.1 mmol L^{-1} β-mercaptoethanol, 2 mmol L^{-1} glutamine, 2 mmol L^{-1} nonessential amino acids, and 1 mmol L^{-1} sodium pyruvate. For mesendodermal differentiation, ES cells were suspended to form aggregates in DMEM medium containing 15% FBS, 0.1 mmol L^{-1} β-mercaptoethanol, 2 mmol L^{-1} glutamine, 2 mmol L^{-1} nonessential amino acids, and 1 mmol L^{-1} sodium pyruvate.

Q-PCR analysis

Cells were dissolved in RNAiso Plus (TaKaRa, China). Total RNA was extracted, and 1 μg of RNA was reverse transcribed using the PrimeScript RT reagent kit (TaKaRa). Quantitative PCR (Q-PCR) was performed using SYBR Premix (BioRad, USA) on a BioRad Q-PCR System. The relative mRNA expression level was calculated using the formula $2^{-\Delta\Delta C_T}$, with *Gapdh* as the normalized control. Real-time primers are listed in Table S1.

Fluorescence-activated cell sorting

Cultured differentiation aggregates were isolated into single cells using 0.05% trypsin-EDTA at 37°C for 2 min. The cells were subsequently strained through a fine-mesh sieve prior to sorting using a FACSC alibur flow cytometer (Becton-Dickinson). ES cells were used as a negative control to set the gates. The FACS data were analyzed using FlowJo (http://www.flowjo.com) software.

Immunofluorescence and alkaline phosphatase staining

Immunofluorescence staining was performed as described previously (Liu et al., 2014). Cultured EBs were digested into single cells using Tryp-LE (Gibco) at 37°C for 2 min. The cells were subsequently replated onto coverslips coated with laminin overnight. Immunofluorescence imaging was performed on a Nikon confocal microscope or animmunofluorescence microscope. The antibodies used for immunostaining are listed in Table S2. For alkaline phosphatase (AP) staining, ES cells with the overexpression or knockdown of *LincRNA1230* were first fixed with 4% paraformaldehyde. AP staining was performed using alkaline phosphatase kits (Sigma, catalog no. 85L3R) according to the manufacturers' instructions.

Lentivirus-mediated gene overexpression and knockdown

For *LincRNA1230* overexpression, *LincRNA1230* was cloned into the pCAG-puroR lentiviral vector. Primers used for LincRNA1230 cloning are listed in Table S3. For *LincRNA1230* knockdown, a shRNA targeting *LincRNA1230* was cloned into the Tet-on PLKO lentiviral vector. The shRNA target sequence is listed in Table S4. Lentivirus

packaging and infection was performed as described previously (Chen et al., 2012). Subsequently, 1 μg mL^{-1} puromycin (Sigma) was added to the ES cell culture medium after two days of infection for a week, and 1 μg mL^{-1} doxycycline (Sigma) was added to the ES cell culture medium to induce the expression of the shRNA targeting *LincRNA1230*.

ChIP analysis

ChIP analysis was performed according to a previously described protocol (Im et al., 2004). A total of 1×10^7 ES cells was digested with trypsin-EDTA and fixed with 1% formaldehyde. Subsequently, 4 μg of each of the antibodies against H3K4me3, H3K27me3 or Wdr5 was added into each sample for the immunoprecipitation of chromatin-associated proteins. ChIP and 10% input DNA was dissolved in 50 μL of ddH_2O after purification. As a template for Q-PCR analysis, 1 μL of ChIP DNA and 1 μL of a 1∶10 dilution of input DNA were used. The fold-enrichments were calculated using the formula $2^{-\Delta\Delta C_T}$. A intergenetic region was used as negative control. The antibodies and primers for ChIP analysis are listed in Tables S5 and S6.

RIP analysis

A total of 1×10^6 ES cells were digested using trypsin-EDTA, washed twice with ice-cold PBS and resuspended in 120 μL of RIP lysis buffer. Approximately 20 μL of magnetic beads were incubated with 4 μL of antibodies against Wdr5, Ezh2, Suz12 or IgG in 100 μL of RIP Wash Buffer for 16 h at 4°C. The beads-antibody complex was subsequently incubated with 100 μL of cell lysate in 900 μL of RIP Immunoprecipitation Buffer for 3 h. After incubation, RNA was purified and dissolved in 10 μL of ddH_2O. A total of 5 μL of RIP RNA and 5 μL of a 1∶10 dilution of 10% input RNA was reverse transcribed. The enrichment of *LincRNA1230* was examined using real-time Q-PCRand subsequently analyzed using the formula $2^{-\Delta\Delta C_T}$. The antibodies used are listed in Table S7.

Western blot

ES cells were digested using trypsin, washed twice with ice-cold PBS, and lysed with RIPA buffer. The protein concentrations were detected using the BCA assay (BioRad) according to the manufacturer's instructions. The antibodies used for western blotting are listed in Table S8.

Statistical analysis

All continuous data are presented as means±SEM. Statistical analyses were performed using unpaired *t*-test. Difference with $P<0.05$ was considered as statistically significant.

Compliance and ethics *The author(s) declare that they have no conflict of interest. All applicable institutional and/or national guidelines for the care and use of animals were followed.*

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SUPPORTING INFORMATION

Figure S1 Schematic diagram shows the location and genomic features of *LincRNA1230*.

Figure S2 *LincRNA1230* doesn't influence the pluripotency maintenance of mouse ES cells.

Table S1 Primers for Q-PCR analysis

Table S2 Antibodies for immunostaining analysis

Table S3 Primers for LincRNA1230 cloning

Table S4 Target Sequence for shLincRNA1230

Table S5 Antibodies for ChIP analysis

Table S6 Q-PCR primers for ChIP analysis

Table S7 Antibodies for RIP analysis

Table S8 Antibodies for Western analysis

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